able since the loss of fluoride ion should be facilitated by the unshared electron pairs of the isopropoxy group. Since isopropoxyfluoromethylene is the only intermediate in the scheme in which the inert end-product, *i*-$PrOCHF_2$, has been by-passed, it must be an intermediate in the formation of the isopropyl orthoformate.

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RECEIVED MARCH 23, 1957

GLUTAMIC ASPARTIC TRANSAMINASE

Sir:

Glutamic aspartic transaminase of pig heart has been highly purified and the presence of firmly bound pyridoxal phosphate has been confirmed. The enzyme was purified by heat denaturation (75° for 20 minutes in maleate buffer *p*H 6.0), ammonium sulfate fractionation (50–67%), adsorption of impurities onto calcium phosphate followed by chromatography on a calcium phosphate column[1] and a repetition of the ammonium sulfate fractionation to give an over-all yield of 30%. Sedimentation and electrophoresis studies indicate that the enzyme is 70% pure; it has an activity of 380 μM glutamate/min./mg. protein using a modification of the assay of Nisonoff, *et al.*,[2] at 37° and *p*H 8.5. The preparation contains one mole of pyridoxal phosphate per 67,000 grams of protein.

Figure 1 shows that the enzyme is colorless at *p*H 8.5 (λ_{max} 362 mμ) but turns yellow at *p*H 4.8 (λ_{max} 430 mμ) and in 0.1 *N* NaOH (λ_{max} 388 mμ). In the latter solution the prosthetic group is split off and could be isolated by use of a Dowex 1-Cl column. In 0.1 *N* HCl the chromophore absorbs strongly at 295 mμ. Its 2,4-dinitrophenylhydrazone, which is not extracted from acidic solution by ethyl acetate, absorbs at 478 mμ in alkali and at 415 mμ in acid. These spectra confirm the fact that the prosthetic group is a derivative of pyridoxal phosphate. It should be noted, however, that the pyridoxal phosphate has characteristically different spectra when bound to the enzyme. We believe that these spectra may be due to a pyridoxal phosphate imine, since Metzler[3] has shown that these imines absorb at 360 mμ at high *p*H and above 410 mμ at lower values. Matsuo and Greenberg[4] have shown that the crystallized cystathionase/homoserine dehydrase has a similar absorption band at 427 mμ.

Carbonyl reagents react with the transaminase to yield the enzyme oxime, hydrazones, cyanhydrin and complexes with thiols and bisulfite, all of which have characteristic spectra.

The addition of glutamate to the enzyme at *p*H 8.5 causes the absorption peak to shift to 332 mμ (see Fig. 1) a peak which is characteristic of all pyridoxine compounds including the zwitterion form of pyridoxamine. Isolation of the prosthetic group on Dowex 1-Cl, after the addition of excess glutamate, yielded pyridoxamine phosphate characterized by its absorption bands in acid (λ_{max} 293) alkali (λ_{max} 306) and at *p*H 5.2 (λ_{max} 326). The addition of less glutamate causes partial disappearance of the pyridoxal phosphate (measured as its 2,4-dinitrophenylhydrazone after cleavage from the enzyme by 0.1 *N* alkali) and the formation of an equivalent amount of α-ketoglutarate.

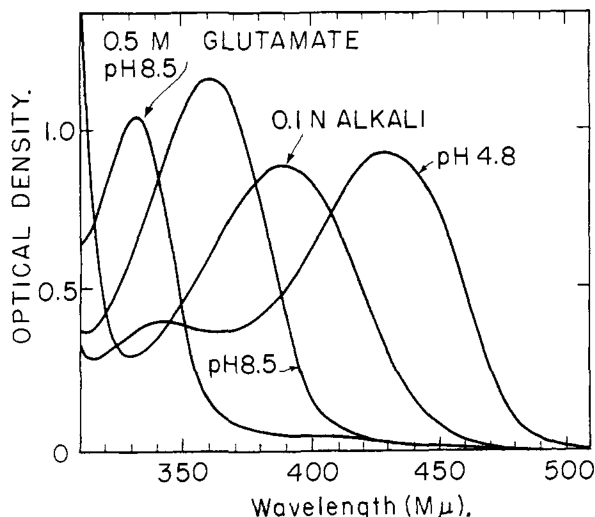

Fig. 1.—Spectra of the enzyme in 0.1 *M* Tris buffer *p*H 8.5 alone and with 0.5 *M* glutamate, in 0.1 *N* NaOH and in 0.15 *M* acetate buffer *p*H 4.8. Each cuvette contained 0.863% protein.

The reaction is reversed by increasing concentrations of ketoglutarate added to the enzyme in the presence of a constant amount of glutamate, as shown by an increase in the band at 362 mμ characteristic of the free enzyme and an increase in the band at 435 mμ ascribed to a complex of ketoglutarate with the protonated enzyme. By the use of C^{14}-glutamate rapid transamination has been confirmed in this steady-state system in which the concentrations of glutamate and ketoglutarate do not change. We believe that these experiments essentially confirm the pyridoxal-pyridoxamine hypothesis suggested on structural grounds by Snell.[5]

Dicarboxylic acids combine with the protonated form of the enzyme to produce an absorption maximum at about 435 mμ. The order of effectiveness is: glutarate > maleate > adipate > malate > ketoglutarate > oxalacetate > succinate. The fact that oxalate, malonate, pimelate, suberate, glycolate and fumarate do not interact suggests that a specific orientation of both carboxyl groups is required and that this specificity may be related to the substrate specificity of the enzyme.

These complexes may explain the apparent stabilizing effect of maleate in this purification and that of ketoglutarate in the purification of kynurenine transaminase.[6] This type of complex formation results in a new type of inhibition,[7] in which the in-

(1) V. E. Price and R. E. Greenfield, *J. Biol. Chem.*, **209**, 363 (1954).
(2) A. Nisonoff, S. S. Henry and F. W. Barnes, Jr., *ibid.*, **199**, 699 (1952).
(3) D. E. Metzler, THIS JOURNAL, **79**, 485 (1957).
(4) Y. Matsuo and D. M. Greenberg, *Federation Proc.*, **16**, 218 (1957).
(5) E. E. Snell, *J. Biol. Chem.*, **154**, 313 (1944).
(6) M. Mason, *Federation Proc.*, **15**, 310 (1956).
(7) A. E. Braunstein, *Nature*, **143**, 609 (1939).

hibitory action of a proton is reinforced by the dicarboxylic acid. Under certain conditions even the keto acid substrates may act as inhibitors rather than activators of the transamination.[8]

The spectral changes with pH may be interpreted as follows. In terms of the studies of Metzler and Snell[3,9] the forms of the pyridoxal imine which may affect the spectra are shown in Fig. 2.

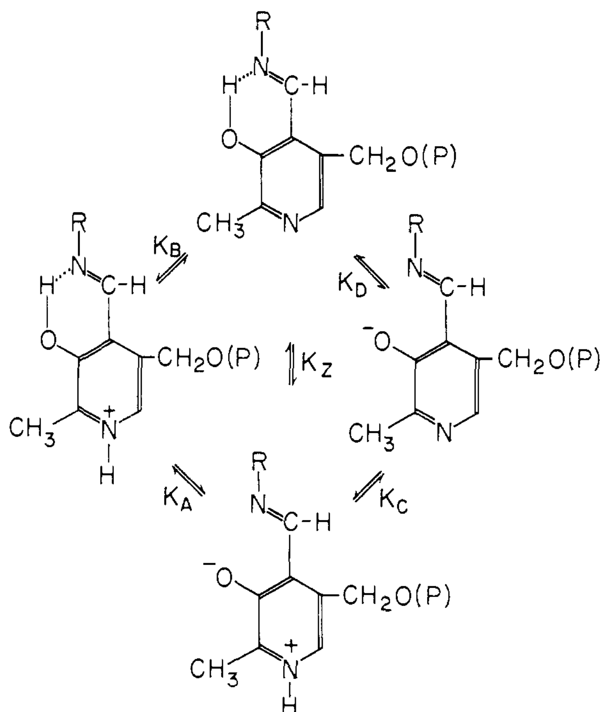

Fig. 2.—The ionization of pyridoxal phosphate imines.

These equations are based on the assumption that the yellow color may be ascribed to the hydrogen chelate ring. Rupture of this ring by a change in pH results in loss of color. A pK_A of 6.2 for this color change for the transaminase suggests that it is the dipolar form of the imine which occurs at neutrality, in contrast to the non-polar forms of the imines studied by Metzler[3] (pK_B = 5.9, pK_D = 10.5).

The implications of these findings with reference to the mechanism of enzymatic transamination will be discussed elsewhere.

Acknowledgment.—We are happy to acknowledge financial support from Ethicon, Inc.

(8) *Cf.* P. Peyser, Doctoral Dissertation, Columbia University, New York, N. Y., 1954.

(9) D. E. Metzler and E. E. Snell, THIS JOURNAL, **77**, 2431 (1955).

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RECEIVED MARCH 21, 1957

A STABLE EQUIMOLAR COPPER(II)-ALBUMIN COMPLEX

Sir:

A simple and rapid amperometric titration technique has been developed for the determination of bovine serum albumin (BSA) with copper(II) in ammoniacal buffer at pH 9.2. Figure 1 shows current voltage curves of copper(II) at the rotated platinum electrode (RPE) in a buffer 0.1 M in ammonia and 0.1 M in ammonium nitrate (curve B). The first wave corresponds to the reduction of copper(II) to copper(I). No copper waves are observed when albumin is present in a molar concentration greater than that of copper (curve D). Curve E illustrates the reappearance of the copper waves when an excess of copper is present.

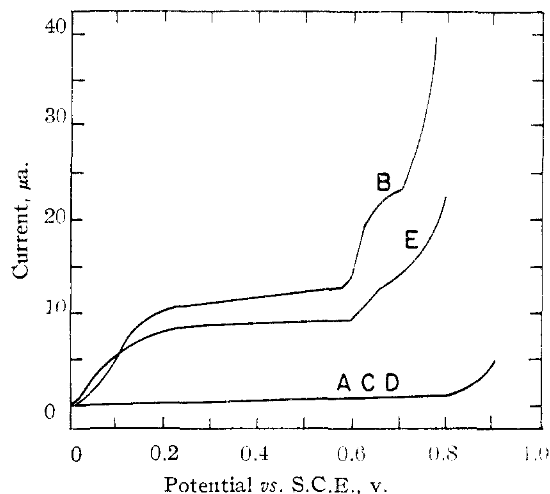

Fig. 1.—Current voltage curves at rotated platinum electrode; buffer, 0.1 M NH_3 + 0.1 M NH_4NO_3; pH 9.2; speed of rotation, 900 r.p.m.: A, residual; B, 4.0 × 10^{-5} M Cu(II); C, 7.3 × 10^{-5} M BSA; D, 7.3 × 10^{-5} M BSA + 6.5 × 10^{-5} M Cu(II); E, 7.3 × 10^{-5} M BSA + 13.0 × 10^{-5} M Cu(II).

By amperometric titration at the RPE in ammonia buffer at −0.4 volt, we have found that copper(II) reacts rapidly with native BSA in a mole ratio of one to one. Similar results have been obtained under proper conditions at pH 8 and 10. The rapid reaction is followed by a slower reaction of additional amounts of copper(II); this does not interfere with the titration. The sulfhydryl group is not involved in the reaction with copper(II). After addition of one or two moles of copper(II) per mole BSA, 0.68 mole sulfhydryl per mole BSA[1] was found by subsequent amperometric titration with mercuric chloride and/or silver nitrate.[2] Also, addition of 0.68 mole of silver nitrate or mercuric chloride per mole BSA prior to titration with copper(II) did not affect the results of this titration.

Titrations of BSA have also been carried out in a denaturing mixture which was 4 M in guanidine hydrochloride, 0.1 M in ammonia and 0.1 M in ammonium nitrate. A reaction ratio of copper(II) to BSA somewhat greater than unity (about 1.3) was found. This higher reaction ratio is ascribed to oxidation by copper(II)[3] of the sulfhydryl group which is oxidizable to disulfide in denatured al-

(1) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947); *Cold Spring Harbor Symposium Quant. Biol.*, **14**, 79 (1949).

(2) I. M. Kolthoff, W. Stricks and L. Morren, *Anal. Chem.*, **26**, 366 (1954); I. M. Kolthoff and W. Stricks, THIS JOURNAL, **72**, 1952 (1950).

(3) I. M. Kolthoff and W. Stricks, *Anal. Chem.*, **23**, 763 (1951).